Omniligase-1-mediated ligation for insulin analog synthesis in solution and on phage surface.

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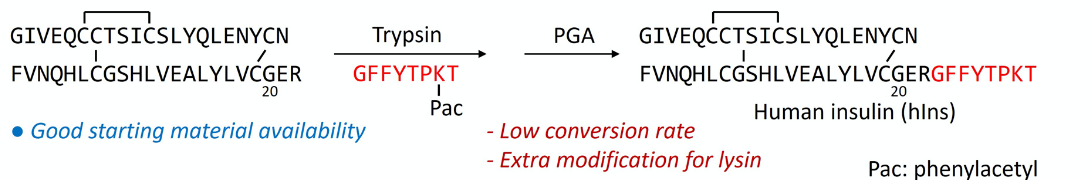
ABSTRACT: The B-chain C-terminal region of insulin has been mutated or modified to achieve improved therapeutic efficacies. For example, all FDA-approved insulin analogs have altered C-terminal segments, which leads to improved pharmacokinetic properties and provide significant clinical benefits on blood sugar regulation. Nonetheless, there is still no efficient method to synthesize insulin analogs with the altered C-terminal region. Herein, we report a facile synthesis using omniligase-1 to ligate an insulin core with a peptide segment in high conversion. We further apply this ligation to M13 phage surface modifications and demonstrate that the phage displayed insulin molecules can bind to insulin receptor ectodomain in an insulin-dependent manner. These results pave the way for building phage display insulin library for therapeutic selections and demonstrate the feasibility of using omniligase-1 to display other disulfide-rich peptides and proteins on phage.

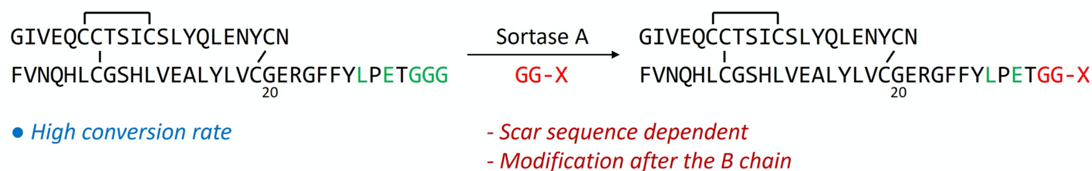
INTRODUCTION

Insulin and its derivatives are life-saving drugs for people with type 1 diabetes (T1D) and some type 2 diabetes (T2D).[1,2] Insulin is a peptide hormone composed of two peptide chains, the A chain (21 amino acids with an intra-chain disulfide bond) and the B chain (30 amino acids), linked together by two disulfide bonds. All U.S. Food and Drug Administration (FDA)-approved insulin analogs to date have modifications on the C-terminal region of the B chain to alter pharmacokinetic properties of insulin for improved glucose controls.[2,3] Additional insulin modifications on the C-terminal region of the B chain were reported to achieve glucose responsiveness,[4–8] aggregation resistance,[9–14] and insulin receptor (IR) isoform selectivity.[15,16] These studies demonstrate that the C-terminal region of the B chain is not critical to insulin bioactivity and can tolerate additional conjugates to some degree, which is supported by the fact that des-B26–30 insulinamide (deletion of B26-30) is fully active.[17] Although modification of the C-terminal region of the B chain has been proved its versatility, compatibility, and reliability for extending the functional scope of insulin, facile methods to fully explore this region is still limited: 1) Acylation on LysB29 has a limited chemical reaction scope.[4,7] 2) Trypsin-mediated ligation using desoctapeptide (B23–30) insulin (DOI) suffers from low conversion rates (Figure 1A).[8,16–19] Our group recently reported sortase A-mediated ligation with a high conversion rate (Figure 1B).[20] However, it can only conjugate peptide substrates after the C-terminus of the B chain. A new way to fully explore the functional scope of the B26–30 region of insulin is highly needed.

A. Prior work: Trypsin



B. Prior work: Sortase A



C. **This work**: Omniligase-1

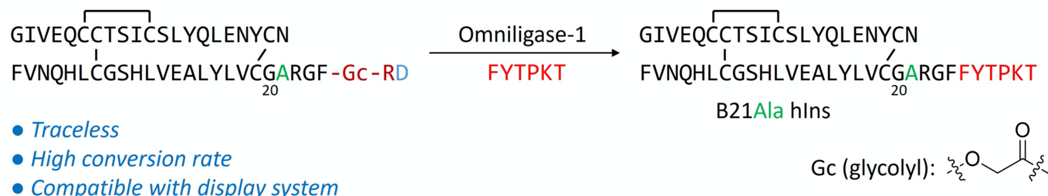


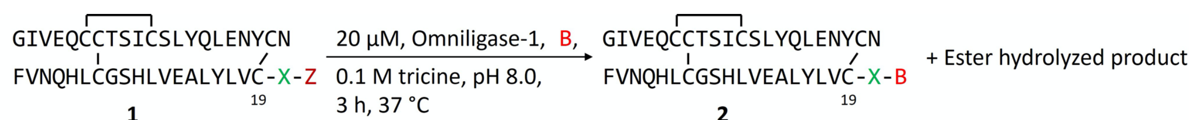
Figure 1. Modification of insulin on the C-terminal region of the B chain through ligation. (A) Trypsin. (B) Sortase A. (C) Omniligase-1.

Omniligase-1, a variant of subtiligase,[21–24] has a broad substrate peptide sequence scope.[25,26] A peptide ester is commonly used as a non-prime side substrate for omniligase-1, and ligated to the N-terminus of another prime-side peptide.[27] Omniligase-1 first uses the thiol group of the catalytic cysteine to activate the ester bond of the substrate peptide ester through transthioesterification. Omniligase-1 then recruits the prime-side peptide and uses its N-terminus to release the enzyme-conjugated peptide through amidation (Scheme S1). Furthermore, omniligase-1 recognizes the P1–P4 residues of the non-prime side peptide and the P1'–P2' residues of the prime-side peptide with various ligation efficiencies.[25,26] We hypothesized that insulin ester with a truncated C-terminal region of the B chain could be ligated with the N-terminus of another peptide by omniligase-1 (Figure 1C) to make insulin in the same manner as sortase A-mediated ligation. If it works, the omniligase-1-mediated ligation will allow modifications on the B26–30 region of insulin with high conversion rates. Herein, we described a synthetic method of insulin through omnniligase-1 ligation between B24–25 with high conversion rates. We further demonstrated that this omniligase-1 approach can be used for phage surface ligation. This work showed the first example of omniligase-1-based phage modification and paved the way of future applications using phage display libraries for ligand selections.

RESUTLS AND DISCUSSION

Evaluation of ligation positions on insulin. We first used published procedures to synthesize insulin cores with an ester on the C-terminal region of the B chain described in this section.[28] To determine the location of ligation, the ester linkage was first employed between B22–B23 of insulin core (**1a**) to test the ligation activity (table 1, entry 1). No ligation was observed after treated with omniligase-1 and the peptide of insulin B23–B30 sequence, GFFYTPKT. The ester of **1a** was hydrolyzed under the basic reaction condition to the same degree regardless of the presence of omniligase-1. According to the sequence recognition preference of omniligase-1, the GluB21 at P2 position of **1a** is a suboptimal residue for omniligation-1 ligation.[26] We mutated GluB21 to Ala to address this issue (Table 1, entry 2) but the ligation product was still not observed for **1b**. We argued that since omniligase-1 needs to recognize P1–P4, the location of ester between B22–B23 may be too close to the bulky disulfide branch at B19, which prevent the ester from entering the catalytic center of omniligase-1. We then moved the ester linkage between B23–B24 (**1c**), which brought two Gly in the sequence to sit at P1 and P4. Both cases are unfavorable for omiligase-1 ligation (Table 1, entry 3).[26] Consequently, the substrate having an ester between B23–B24 (**1c**) was omitted in this study. We next turned to an ester linkage between B24–B25. The insulin ester with human sequence and an ester between B24–B25 (**1d**) was predicted no ligation activity because B21Glu at P4 was reported forbidden for omniligase-1 ligation.[26] As predicted, no ligation was observed for **1d** (table 1, entry 4). Mutation of GluB21 to Ala of insulin was reported having low influence on the binding affinity to IR[29] and, importantly, B21Ala at P4 is an allowed residue for omniligase-1 ligation.[26] The insulin ester with B21Ala mutation and an ester between B24–B25 (**1e**) was successfully ligated with the peptide of insulin B25–B30 sequence, FYTPKT, by omniligase-1 to give the B21Ala insulin analog (**2**) at 37°C at 3 hours with a 70% ligation rate (table 1, entry 5). However, **1e** was not completely soluble at 0.5 mM working concentration in the ligation buffer. **1e** could be completely dissolved in water and prepared as a stock solution in a high concentration (3–5 mM) but was immediately precipitated after added into the ligation buffer at pH 8.0. The precipitant **1e** was later consumed gradually along with the reaction proceeding. We proposed that the Glu to Ala mutation led to reduced hydrophilicity. This mutation also increased the isoelectric point (pI) of insulin to be closer to the buffer pH and therefore made **1e** less soluble than **1d**. Adding an extra Asp at the C-terminus of **1e** (**1f**) could decrease the pI and compensate the loss of hydrophilicity to rescue the solubility and yield the same ligation product **2**. **1f** was completely soluble in the ligation buffer as a 0.5 mM solution and could be ligated with the peptide of insulin B25–B30 sequence, FYTPKT, by omniligase-1 to give insulin analog **2** with a mg-scale isolation yield of 77% (Table 1, entry 6, Figure S1). Due to the excellent result, insulin core **1f** was selected for subsequent studies using omniligase-1.

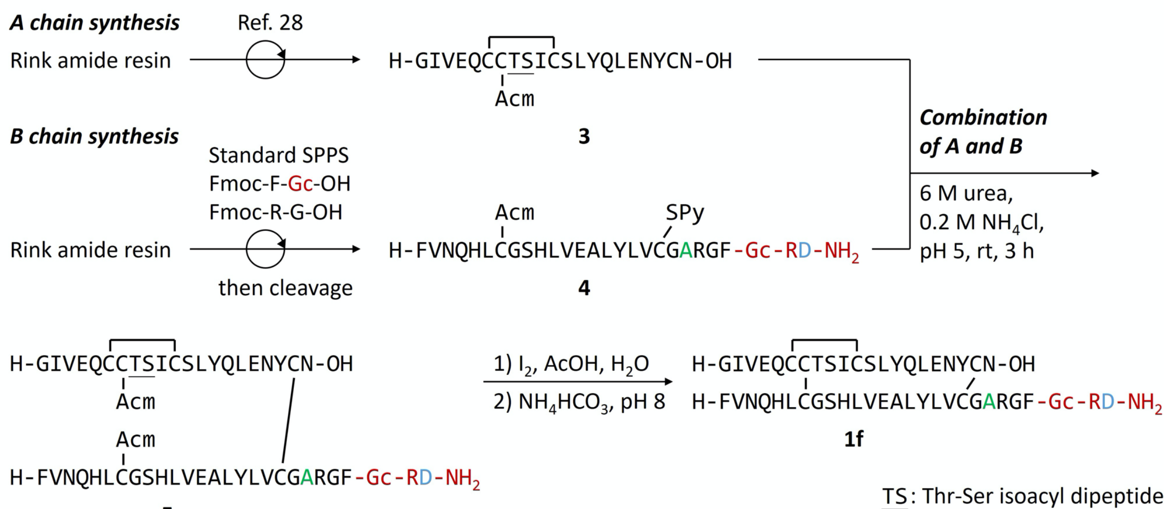
Table 1. Investigation of insulin ester substrates for omniligase-1 ligation.



Entry	Substrate[a]	Gc location	X	Z	B	Product	Ligation (%)
1	**1a**	B23	GER	Gc-R-NH_2	GFFYTPKT	hIns	n.d.[b]
2	**1b**	B23	GAR	Gc-R-NH_2	GFFYTPKT	**2**	n.d.[b]
3[c]	**1c**	B24	GERG	Gc-R-NH_2	FFYTPKT	hIns	-
4	**1d**	B25	GERGF	Gc-R-NH_2	FYTPKT	hIns	n.d.[b]
5[d]	**1e**	B25	GARGF	Gc-R-NH_2	FYTPKT	**2**	70
6	**1f**	B25	GARGF	Gc-RD-NH_2	FYTPKT	**2**	77[e]

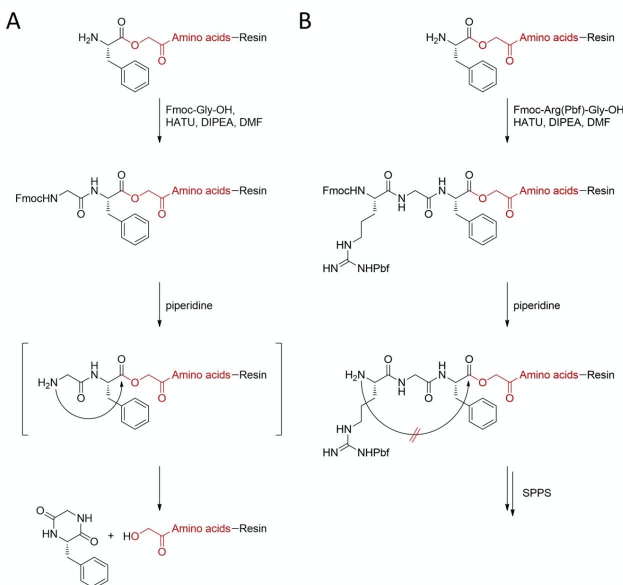
[a]0.5 mM. [b]n.d. = not detected at 24 h. [c]**1c** was not tested because sequence X (GERG) is not compatible with omniligase-1. [d]Solubility < 0.2 mM. [e]Isolation yield for mg-scale.

Scheme 1. Synthesis of **1f**.

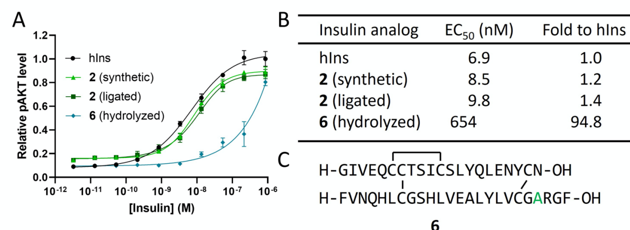


Synthesis of insulin 1f. The synthesis of insulin core **1f** was further optimized using a modified strategy (Scheme 1).[28] The insulin A chain **3** and B chain **4** were independently synthesized through solid-phase peptide synthesis (SPPS) followed by chain combination. The A chain was processed as previously reported[28] to give the A6-A11 disulfide, A7 Cys(Acm), and A20 free Cys A chain **3**. To introduce the ester function group on the B chain **4**, glycolyl (Gc) group was incorporated as a pre-made Fmoc-Phe-Gc-OH building block used in the SPPS by following the reported procedure (Scheme S2).[30] In short, Fmoc-Phe-OH was linked with benzyl bromoacetate to give Fmoc-Phe-Gc-OBn followed by hydrogenolysis of the benzyl ether protecting group to give Fmoc-Phe-Gc-OH. However, during the preparation of the B chain **4**, a significant amount of GlyB23-PheB24 dipeptide elimination was observed. This deletion has been reported through diketopiperazine (DKP) elimination occurring after the N-terminal Fmoc of GlyB23 was deprotected during the SPPS (Scheme 2A).[31] To prevent the DKP elimination and increase the yield of **4**, Fmoc-Arg(Pbf)-Gly-OH dipeptide as a building block was used to avoid creating the N-terminal amine of GlyB23 (Scheme 2B). Fmoc-Arg(Pbf)-Gly-OH was prepared from the coupling of Fmoc-Arg(Pbf)-OH and H-Gly-OBn followed by hydrogenolysis of the benzyl ether protecting group (Scheme S3). Cys(Acm) was used at B7 for the A7-B7 disulfide. Cys(Trt) was used at B19 and activated with 2,2'-dithiodipyridine (DTDP) during peptide cleavage for the A20-B19 disulfide at the A chain and B chain combination step. After the A chain **3** and B chain **4** were prepared, two chains were combined in equal amounts under pH 5.0 at room temperature for 3 hours to form the A20-B19 disulfide bond of **5**. The third disulfide between A7 and B7 was formed by the treatment of iodine under an acidic condition. The Thr-Ser isoacyl linkage was finally transformed to an amide bond through an *O*-to-*N* acyl shift under pH 8.0 to give insulin ester **1f** (Scheme 1).

Scheme 2. (A) The DKP elimination. (B) Using Fmoc-Arg(Pbf)-Gly-OH dipeptide to prevent the DKP elimination.



Bioactivity test. Upon the success of ligation between **1f** and the peptide FYTKPT to give insulin analog **2**, the activity of ligated **2** was characterized through a cell-based assay by using pAKT levels as measurements of IR downstream signaling (Figure 2). Noted, insulin analog **2**, which has a single Glu to Ala mutation at B21 position, was previously reported to have similar binding affinity as hIns to IR.[29] We showed that the ligated **2** had a comparable EC_{50} with recombinant hIns from a commercial source, as well as the same analog **2** from the completely synthetic full B chain by SPPS (Scheme S4).[28]



Insulin analog	EC_{50} (nM)	Fold to hIns
hIns	6.9	1.0
2 (synthetic)	8.5	1.2
2 (ligated)	9.8	1.4
6 (hydrolyzed)	654	94.8

Figure 2. (A) *In vitro* activity of hIns, synthetic **2**, ligated **2**, and hydrolyzed **6** by using activated pAKT levels as measurements. Data are expressed as mean ± SD (n = 4 per group). (B) EC_{50} hIns, synthetic **2**, ligated **2**, and hydrolyzed **6** calculated by Prism 9 (GraphPad Software, California, USA) with nonlinear regression curve fitting of dose-response asymmetric equation. (C) The hydrolyzed byproduct 6 from 1f during the ligation reaction.

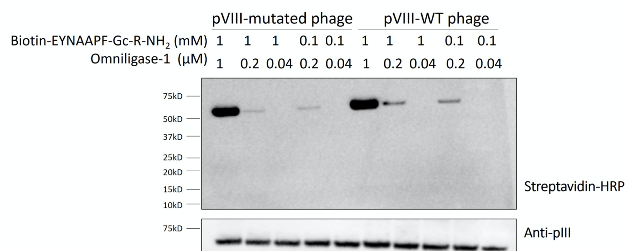


Figure 3. Omniligase-1 mediates selective ligation on pIII. pVIII-WT phage and Glu to Pro pVIII-mutated phage were incubated with different concentrations of omniligase-1 and biotin-EYNAAPF-Gc-R-NH_2 for 20 h at 37 ºC with 0.1 M tricine at pH 8.5. The ligation was monitored by western-blot using streptavidin-HRP and anti-pIII antibody. Omniligase-1 could selectively and specifically mediate the ligation of pIII regardless the sequence of pVIII. 1 mM biotin-EYNAAPF-Gc-R-NH_2 and 1 μM omniligase-1 generated the highest ligation efficiency.

The hydrolyzed byproduct **6** from **1f** during the ligation reaction had a nearly 100-fold decreased activity compared to hIns and **2**, which is consistent with the previous reports that PheB25 is critical for receptor engagement.[32,33]

Omniligase-1-mediated selective M13 phage labeling. Tremendous progress was made in expanding the phage display library with synthetic functional groups by chemical or chem-enzymatic reactions.[34–37] There are two main M13 phage surface proteins that can be used for peptide modifications, pIII and pVIII. pIII-library is the preferred version for high affinity ligands due to its lower copy number (5 vs up to 2700 for pVIII). Unlike previous examples such as phage libraries generated from sortase ligations where a specific epitope is required for the ligase,[35] omniligase-1 has a broader substrate scope, which will further enhance the library diversity. However, this also creates a new selectivity challenge because both pIII and pVIII can potentially be modified. To avoid pVIII modifications (Figure S2), we first introduced a Glu to Pro mutation in the second residue of pVIII with FYTPKT displayed at the N-terminus of pIII. We hypothesize that this can prevent any pVIII ligations because Pro at P2' is a disallowed substrate for omniligase-1. This pVIII-mutated phage was incubated with different concentrations of omniligase-1 and a biotin-labeled substrate (biotin-EYNAAPF-Gc-R-NH_2),[22–24] which allow us to monitor the ligation using streptavidin-HRP. After 20 hours at 37 ºC (Figure 3), the biotin-containing band was only detected at 55 kDa, which is the same molecular weight as pIII. We did not observe any pVIII modifications. We also performed the same ligation reaction using the pVIII-wild type (WT) phage clone. To our surprise, there was no difference between the two phage clones, and no streptavidin signal was detected at pVIII size, even for the phage with wild type pVIII. The result indicated that the omniligase-1-mediated ligation was selective toward pIII. To our best knowledge, this is the first study using omniligase-1 for phage display. Although pVIII has a much higher copy number, omniligase-1 still preferentially reacted with pIII likely due to steric hindrance of the environment surrounding pVIII.

Omniligase-1-mediated insulin display on pIII. To further explore omniligase-1-mediated pIII labeling, phage clone with FYTPKT displayed at the N-terminus of pIII was incubated with different concentrations of insulin ester **1f** and omniligase-1 for 20 h at 37 ºC or room temperature (Figure 4A). The reaction was monitored by immunoblot using anti-pIII antibody and anti-hIns antibody. From the blots (Figure 4B), a new pIII band was detected around ~65 kDa, corresponding to the insulin-pIII fusion product. We found the ligation efficiency was higher at room temperature than 37 ºC. The lower amount of insulin-pIII adduct in 37 ºC indicated that pIII was proteolyzed by omniligase-1 due to the protease activity of the enzyme. We further evaluated the reactions at even lower temperatures (4 ºC and 12 ºC) (Figure S3) and found that room temperature led to the highest ligation efficiency with 20 μM omniligase-1 and 2 mM insulin ester **1f** in which ~50% pIII was successfully ligated with insulin. A higher concentration of omniligase-1 (80 μM) did not generate even higher efficiency.

After identifying the optimized ligation condition, phage clone with FYTPKT at N-terminus of pIII was incubated with 20 μM omniligase-1 and 2 mM insulin ester **1f** for 20 h at room temperature. After verifying the success of ligation by immunoblot with anti-hIns antibody (Figure S4), the phage clones were purified by two rounds of PEG participation to remove excess **1f** in the solution. pIII is critical to infect its bacterial host for amplification and this ability may be sabotaged with a modified pIII. We showed that omniligase-1-mediated insulin ligation did not affect its viability (Figure S4). To further investigate the biological function of ligated insulin on phage, 10^9 phage were incubated with magnetic beads, which were precoated with biotinylated IR ectodomain or biotin along. After rounds of wash to remove unbound phage by 0.1% PBST, the bound phage was eluted, and the yield of binding was determined by phage titration. The insulin-displaying phage had the highest yield (~20%) for positive binding signals, and all other control groups only had background signals (Figure 4C). We further performed a competitive binding assay using hIns as a soluble competitor. With increased concentrations of hIns, the percentage of phage binding to IR decreased, showing a dose-dependent IR-specific binding (Figure 4D). These results indicated that insulin ester **1f** can be selectively ligated on phage pIII using omniligase-1. Furthermore, the displayed insulin can bind to its binding receptor in an insulin-dependent manner. Previously, we reported the use of a yeast display system to display single-chain insulin analogs with functional properties.[38] However, a two-chain insulin cannot be achieved due to the complication of insulin C-peptide removal. To our best knowledge, this is the first demonstration of a two-chain insulin in a display system that demonstrated functional properties. This omniligase-1-mediated ligation also paves the way for displaying other disulfide-rich peptides or proteins on phage.

CONCLUSION

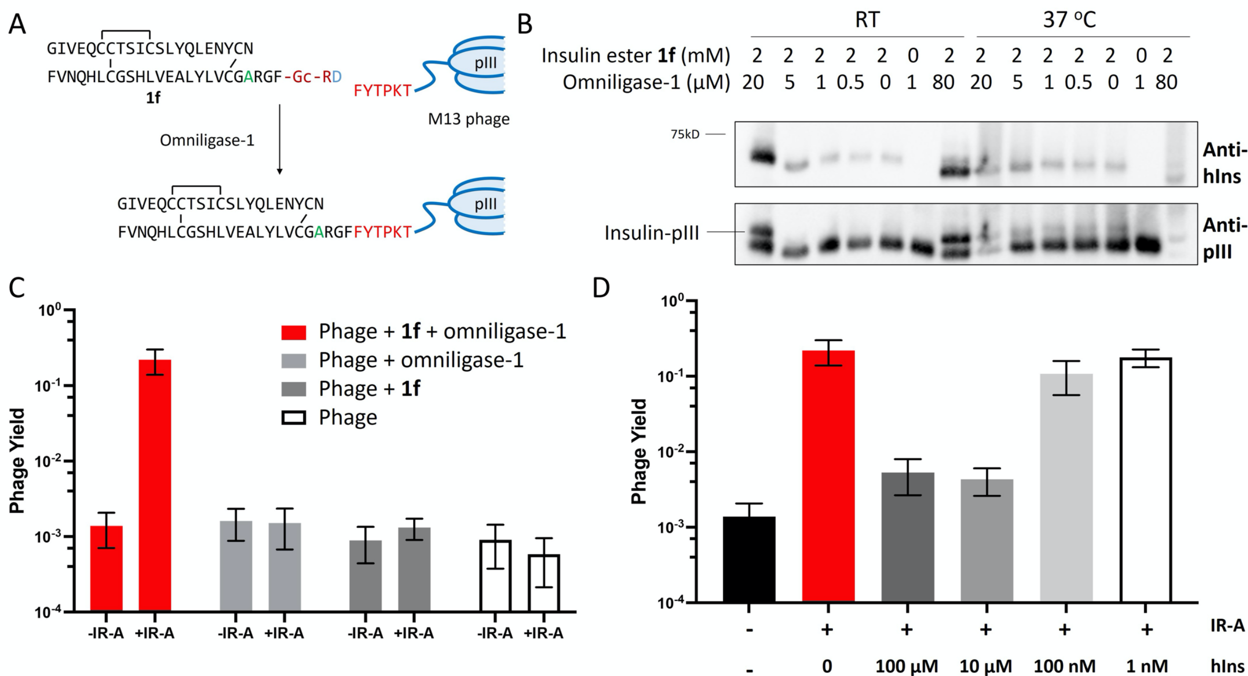


Figure 4. Omniligase-1 mediates insulin ligation on pIII. (A) Schematic representation of insulin ligation with pIII mediated by omniligase-1. (B) FYTPKT-pIII modified phage was incubated with different concentrations of insulin ester **1f** and omniligase-1 for 20 h at 37 °C or room temperature with 0.1 M tricine at pH 8.5. After the reactions were finished, western blot was conducted under non-reducing condition, and anti-hIns antibody and anti-pIII antibody were used to monitor the reactions. Compared with 37 °C, room temperature had an optimize ligation efficiency and around 50% of pIII were modified with insulin under the ligation condition with 2 mM insulin ester **1f** and 20 µM omniligase-1. (C) Phage yield after IR pulldown assay. The binding assay was conducted with the insulin-displaying phage, phage after treatment of omniligase-1 only, phage after treatment of insulin ester **1f** only, and phage clone only. After incubation with IR on magnetic beads (+IR-A), the mixture was washed, bound phage was eluted, and phage titer was determined by top agar. The magnetic beads without IR (-IR-A) were used as a negative control. Data are expressed as mean ± SD (n = 4 per group). (D) Phage yield after competitive binding assay with hIns. The insulin-displaying phage was incubated with increased concentrations of hIns. After incubation, washing, and elution, the phage yield was determined by top agar. Data are expressed as mean ± SD (n = 4 per group).

We reported a synthetic method of insulin through the traceless omniligase-1-mediated ligation between a peptide (FYTPKT) as the B-chain C-terminal region and an ester-containing insulin core (**1f**) with a high conversion rate. The resulting ligated B21Ala hIns (**2**) had a comparable cell-based IR-stimulative signaling potency with the commercial recombinant hIns. The insulin ester **1f** was able to be selectively conjugated to M13 phage pIII expressing sequence FYTPKT at the N-terminus by omniligase-1 without observable insulin-pVIII conjugates and with other phage proteins. The phage displayed insulin on pIII could bind to IR ectodomain in an insulin-dependent manner. To the best of our knowledge, this was the first demonstration of a two-chain insulin in a display system that presented functional properties. These results established a foundation of generating phage display insulin library for therapeutic selections and demonstrated the potential for omniligase-1 to display other disulfide-rich peptides and proteins on phage.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Detailed synthetic protocols, experimental methods and characterization data are included.

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The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / ‡These authors contributed equally.

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Notes

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ABBREVIATIONS

DKP, diketopiperazine; DOI, desoctapeptide (B23–30) insulin; DTDP, 2,2'-dithiodipyridine; FDA, U.S. Food and Drug Administration; Gc, glycolyl; hIns, human insulin; IR, insulin receptor; pI, isoelectric point; SPPS, solid-phase peptide synthesis; T1D, type I diabetes; T2D, type II diabetes; WT, wild type.

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Graphical Abstract